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(54) **Biotinylated monoclonal antibodies, avidin and biotin for diagnosis and therapy.**

(57) A product in the form of a combined preparation of reagents for sequential administration to a mammal suffering or suspected to be suffering from solid tumours for the diagnosis and/or treatment of such disorders includes (a) a biotinylated monoclonal antibody (or a fragment thereof) specific to a tumour-associated antigen and able to persist on the surface of the tumour for a period of not less than 48 hours, (b) a protein of the avidin type which can bind specifically to biotin, (c) biotin, or derivatives thereof, conjugated or intended to be conjugated with an agent selected from radioisotopes, paramagnetic agents and cytotoxic agents and, preferably, (d) a

biotinylated agent capable or remaining in the plasma for a certain period of time and capable of binding and removing the circulating avidin which is not fixed to the tumour cells.

The present invention relates to a preparation for the diagnosis and treatment of tumours and to its use for these purposes. More particularly, the invention relates to a combination or kit including reagents for sequential administration in order to fix selectively radioactive isotopes, cytotoxic drugs or paramagnetic agents to tumour cells in a mammal.

It is known that murine monoclonal antibodies specific to tumour-associated antigens expressed by human tumours have been used, after radiolabelling with suitable radioisotopes, for diagnostic purposes (radioimmunoscintigraphy) or for therapeutic purposes (radioimmunotherapy).

The diagnostic technique is based on the assumption that the aforesaid antibodies can localise on the tumour cells with sufficient selectivity to enable them to be visualised by a gamma camera. It has been found, however, that some of the antibodies are localised non-specifically, mainly in the liver, the spleen and the bone marrow. This disadvantage limits the usefulness of this diagnostic procedure because the results can only be interpreted correctly by operators who are very familiar with it, since complex technique are needed to differentiate between specific and non-specific localisations.

The use of radiolabelled monoclonal antibodies for therapeutic purposes has also been proposed but it has been found that it is not possible, in practice, to deliver a high enough dose of radioactivity (about 5000 rads) to the tumour to destroy it selectively, except when intraperitoneal or intrapleural tumours are treated by intracavitory *in loco* administration. When the radioactive monoclonal antibodies are administered intravenously, however, the maximum dose of radioactivity which can be delivered to the tumour without causing intolerable damage to the whole body system, is considerably less.

Within the scope of the present invention, it has been found that, when monoclonal antibodies which recognise a specific tumour-associated human antigen are first conjugated with biotin, then labelled and then administered to man by systemic or intracavitory way, their fate is similar to that of the corresponding non-biotinylated radioactive monoclonal antibodies. At any rate, they are localised mainly on the tumour cells and, to a lesser extent, at non-specific sites; they then persist on the surfaces of the tumour cells for many days whilst, at the non-specific localisations, they are internalised and catabolised by the cells within about 24-48 hours. At an appropriate time after administration, therefore, the only extracellular biotins are those anchored to the tumour cells, whilst all the biotin naturally present in the human body (vitamin H) and the exogenous biotin conjugated with that fraction of the monoclonal anti-

bodies which, after administration, reached non-specific sites are located within cells and hence are no longer directly accessible to extracellular fluids.

5 The fact that the monoclonal antibody which is internalised at the sites of non-specific localisation is biotinylated but non radioactive has the advantage of preventing the radiation damage which is normally caused when radiolabelled antibodies are internalised after *in vivo* administration.

10 A further observation upon which the present invention is based is that, although avidin is a heterologous protein, it does not give rise to toxicity or immediate hypersensitivity in man and does not show non-specific localisations.

15 A subject of the present invention, therefore, is a product in the form of a combined preparation of reagents for sequential administration to a mammal suffering or suspected to be suffering from a solid tumour for the diagnosis and/or treatment of such disorders, characterised in that it includes:

20 a) a biotinylated monoclonal antibody, or biotinylated fragments thereof, specific to a tumour-associated antigen expressed by the tumour and able to persist on the surface of the tumour for a period of not less than 48 hours;

25 b) a protein of the avidin type which can bind specifically to biotin, and

30 c) biotin, or derivatives thereof, conjugated or intended to be conjugated with an agent selected from the group consisting of radioisotopes, cytotoxic agents and paramagnetic agents.

35 The sequential use of these reagents, which enables a radioactive, cytotoxic or paramagnetic agent to be fixed selectively to tumour cells, includes the steps of:

40 i) administering a measured quantity of the component a) intravenously,

45 ii) administering a measured quantity of component b) intravenously after a suitable period of time following the administration of the component a), the period of time being long enough for the biotinylated monoclonal antibodies which are not fixed to the tumour as a tumour-associated antigen/biotinylate antibody complex to be catabolised, and

50 iii) administering a measured quantity of the component c) intravenously, at least 24 hours (preferably 48 hours) after the administration of the component b).

55 Advantageously, the preparation also includes a biotinylated agent d) capable of remaining in the plasma for a certain period of time and capable of binding and removing the circulating avidin not fixed to the tumour cells. This component may be constituted, for example, by biotinylated human albumin or an equivalent serum protein such as biotinylated transferrin or biotinylated im-

munoglobulin IgG.

In this preferred embodiment, the administration of the component c) is preceded, by a few minutes, (from 3 to 15 minutes), by injection of a quantity of the component d) substantially equimolar to the quantity of circulating avidin, which is determined beforehand by known methods.

Suitable monoclonal antibodies usable within the scope of the present invention are those which remain on the external surfaces of tumour cells for a sufficient period of time (4-20 days) and anyway for at least 48 hours. This ability to persist on the external surfaces of the tumour cells has to be determined beforehand for each antibody by tests on animal models and tests carried out by known techniques on bioptic or autoptic samples and then subsequently confirmed on bioptic samples resulting from clinical investigations in man.

Examples of suitable monoclonal antibodies are the following murine monoclonal antibodies available from Sorin Biomedica, Saluggia (VC), Italy:

- FO23C5 (directed towards CEA antigen which is expressed by carcinomas of various histotypes),
- B 72.3 (directed towards TAG-72 antigen which is expressed by epithelial carcinomas),
- 255.28S (directed towards HMW-MAA antigen which is expressed by malignant melanoma).

In the known diagnostic use (immunoscintigraphy), these antibodies are usually labelled, by known techniques, with radioisotopes emitting solely, or mainly, gamma radiation.

Examples of such radioisotopes are Indium-111, Technetium-99m, Iodine-123 and Iodine-131. The latter, which also emits beta radiation, is also used for therapeutic purposes. However, as already indicated above, when administered intravenously, these radiolabelled monoclonal antibodies cannot concentrate the radiation dose necessary to destroy the tumour (at least 5000 rads) without at the same time causing systemic damage to the patient as a result of unwanted radiation to normal tissues and cells.

In the diagnostic or therapeutic use according to the present invention, the monoclonal antibodies specific to tumour-associated human antigens which can remain on the external surfaces of the tumour cells for at least 48 hours are not administered in the form of radiolabelled antibodies. They are treated, instead, with reactive biotin derivatives (such as, for example, esters) prepared according to known techniques so as to form a compound in which biotin and the monoclonal antibody are conjugated by a covalent bond. For this purpose, one of the various commercial compounds indicated as

suitable for biotinylating proteins, such as, for example, ENZOTIN^(R) from Enzo Biochemicals, N.Y., U.S.A., or BIOTIN X NHS from Società Prodotti Antibiotici of Milan, may conveniently be used.

5 The solution of the biotinylated monoclonal antibody thus obtained is dialysed against a sterile physiological solution and then sterilised by filtration through suitable cellulose filters with pores of 0.22 microns. After its sterility, apyrogenicity and all 10 the characteristics required for an injectable solution have been checked, the solution thus obtained is administered to the patient.

15 The biotinylated monoclonal antibodies are stable and are therefore suitable for commercial preparations in large batches according to known techniques. If reagents are to be supplied in a commercial kit form, bottles containing the monoclonal antibody either in the form of freeze-dried powder or 20 as a sterile pyrogen-free solution ready for use are provided.

25 The quantity of biotinylated monoclonal antibodies injected will vary according to parameters known in the art, such as, for example, the body weight of the patient, his physiopathological conditions and the purpose (diagnostic or therapeutic) to be achieved. Usually, however, it will vary between 0.007 and 0.14 mg/kg and, in particular, 0.007-0.028 mg/kg for diagnostic use and 0.028-0.14 for therapeutic use. Any overdose has contraindications for the patient and does not, therefore, 30 comprise the subsequent stages since any excess of the biotinylated antibody will be eliminated, according to the present invention, by the subsequent administration of avidin. The necessary time must 35 elapse between the administration of the biotinylated monoclonal antibody and the administration of the avidin for the biotinylated monoclonal antibodies which are localised at non-specific sites to 40 be internalised and catabolised by the non-tumorous cells to which they are bound. This time will be at least 24 hours, preferably 48 hours.

45 The general term avidin indicates a group of proteins which are characterised, from a functional point of view, by the property that they bind biotin (formerly called vitamin H) with a very high affinity and specificity. They are produced by various higher organisms (birds, amphibians, etc.), wherein 50 they are present in the eggs, or by a Streptomyces (*Streptomyces avidinii*). The former group of proteins are strongly basic glyco-proteins known as avidin in the strict sense; the latter is not a glycoprotein, has an isoelectric point near neutrality, and is known by the term of streptavidin.

55 These proteins are commercially available either in the native form or modified as a result of partial proteolysis (like the so-called core streptavidin), partial deglycosylation or chemical modification (acetylavidin, succinylavidin, etc.).

All these proteins are their derivatives, although not explicitly mentioned, will be covered by the term avidin in the present description and in the claims.

Advantageously, the injection with avidin is carried out in two successive steps: 1/5 of the dose added to 3 ml of physiological solution injected rapidly in a bolus to favour the clearance of the circulating biotinylated antibodies from the plasma, and the remaining 4/5 in 100 ml of physiological solution administered slowly (over a period of 15-30 minutes) after 5-15 minutes from the first injection. Advantageously, the kit thus includes two separate measured quantities of avidin in the proportions indicated above.

The total dose of avidin is intended to represent a molar excess of at least 10 times and at most 100 times the quantity of antibodies injected. The dose is generally between 0.07 and 1.4 mg/kg and, in particular, 0.07-0.14 mg/kg for diagnostic use and 0.7-1.4 mg/kg for therapeutic use.

The method according to the present invention provides for the administration of avidin which is not radiolabelled (referred to below as "cold" avidin) for two distinct purposes which are both advantageous for diagnostic and/or therapeutic purposes: 1) the avidin eliminates the circulating biotinylated antibodies from the circulating plasma and concentrates them in organ such as the liver and the spleen where they are rapidly catabolised; 2) avidin enables a large quantity of a radioactive, cytotoxic or paramagnetic agent to be concentrated on the surface of the tumour as a result of the subsequent administration of biotin, with a less harmful effect on the entire organism. In fact, given that avidin can bind up to 4 units of biotin, the tumour-associated antigen/biotinylated monoclonal antibody/avidin complex will have a terminal avidin unit which can capture and fix up to 3 molecules of the biotin derivative c) mentioned above.

A preferred and characterising aspect of the present invention is the fact that the administration of a radioactive, cytotoxic or paramagnetic derivative of biotin is preceded by a few minutes, by the administration of a biotinylated agent capable of remaining in the plasma for a certain period of time and capable of binding and removing the circulating avidin not fixed to the tumour cells, such as, for example, biotinylated human albumin or an equivalent agent such a biotinylated transferrin or biotinylated immunoglobulin IgG.

The quantity of biotinylated albumin administered will be equimolar to the quantity of avidin circulating at the time of the injection. The avidin circulating is measured according to methods known to an expert in the art. This administration has the function of leading to the formation of circulating avidin-biotinylated albumin complexes,

thus saturating the biotin-binding sites still present in the blood. These complexes are then captured by the liver and the spleen so that their level in the circulation is reduced. The formation of circulating complexes of avidin with radiolabelled biotin or biotin conjugated to cytotoxic or paramagnetic agents is thus reduced to minimum values thus significantly reducing the background due to these complexes circulating in the blood, particularly during the immunoscintigraphic or NMR determination.

The biotin or its derivative c) is administered after a period of at least 24 hours, and preferably 48 hours, in doses of from 2.8 to 70 µg/kg and, in particular, within the range 2.8-7 µg/kg for diagnostic use and 7-70 µg/kg for therapeutic use.

Preferably, radioisotopes selected from group a) and b) below and intended for diagnostic use and therapeutic use, respectively, are used for the preparation of the radiolabelled compound: a) Fe-52, Mn-52m, Co-55, Cu-64, Ga-67, Ga-68, Tc-99m, In-111, I-123, I-125, I-131; b) P-32, Sc-47, Cu-67, Y-90, Pd-109, Ag-111, I-131, Pm-149, Re-186, Re-188, At-211, Pb-212, Bi-212.

Within the scope of the invention, the biotin and/or derivatives thereof may also be conjugated with paramagnetic agents, such as gadolinium, iron, manganese and the like, for use in the nuclear magnetic resonance method.

Moreover, biotin and derivatives thereof may be conjugated with non-radioactive cytotoxic substances, such as ricin, Adriamycin, cis-platinum and similar chemotherapeutic agents, which are directly conjugated with the biotin or contained in biotinylated liposomes.

In the selection of a suitable radioisotope, account will be taken of known criteria such as the type of radiation emitted by the radionuclide and the purpose (diagnostic or therapeutic) to be achieved by the administration of the radioactive compound. The biotin or derivative thereof will be radiolabelled according to known techniques which produce a stable compound.

The same considerations apply to the conjugation with cytotoxic or paramagnetic agents.

The main characteristic of the radioactive biotin derivative is its low molecular weight which results in a biological half-life shorter than that of a radiolabelled antibody and which thus enables large doses of radioactivity to be administered with relatively low systemic radiation doses.

Another characteristic of the present invention is the use of "cold" avidin and "hot" biotin or a paramagnetic or cytotoxic derivative of biotin in order to fix selectively suitable radioisotopes or other agents to tumour cells in man.

Another characteristic of the present inventions is constituted by the use of the avidin-biotin system described above for detecting tumorous lesions

during surgical operations for the resection of the tumour. This detection may be carried out with the use of a suitable radioactivity detection probe and suitable specific counting instrumentation, such as that supplied by Neoprobe Inc., Columbus, Ohio, U.S.A. The method could provide for the administration of cold biotinylated antibodies followed by injection of avidin and biotinylated albumin in the quantities and at the intervals described above and by the administration of biotin radiolabelled with I-125, I-123 or Tc-99m between 2 and 24 hours before the operation.

Suitable kits containing measured quantities of the components a), b), c) and, preferably d), which constitute a further subject of the present invention, may be produced commercially to enable the avidin-biotin system described to be used routinely *in vivo*.

A kit according to the invention typically includes:

- a bottle (or vial) containing from 0.5 to 10 mg of at least one biotinylated monoclonal antibody a),
- a bottle containing from 5 to 100 mg of avidin b) or two bottles containing 1/5 and 4/5 of the total dose, respectively,
- a bottle containing from 0.1 to 5 mg of biotin or a derivative thereof c), and
- preferably, a bottle of biotinylated albumin d) or an equivalent agent as described above, in a quantity of from 4 to 20 mg of injectable solution.

The components a), b), c) and d) may be in the form of a sterile and pyrogen-free aqueous solutions having the characteristics required for injectable products or, alternatively, in the form of sterile and pyrogen-free freeze-dried powders. In this case, the kit also includes one or more bottles containing 10-100 ml of suitable sterile and pyrogen-free injectable solutions, which may even be of different kinds but are such as to enable the preparations supplied as freeze-dried powders to be reconstituted.

Claims

1. A product in the form of a combined preparation of reagents for sequential administration to a mammal suffering or suspected to be suffering from solid tumours for the diagnosis and/or treatment of such disorders, characterised in that it includes:
 - a) a biotinylated monoclonal antibody (or a fragment thereof) specific to a tumour-associated antigen and able to persist on the surface of the tumour for a period of not less than 48 hours,
 - b) a protein of the avidin type which can

bind specifically to biotin,
c) biotin, or derivatives thereof, conjugated or intended to be conjugated with an agent selected from radioisotopes, paramagnetic agents and cytotoxic agents.

5. 2. A product according to Claim 1, also including:
 - d) a biotinylated agent capable of remaining in the plasma for a certain period of time and capable of binding and removing circulating avidin not fixed to the tumour cells such an agent being selected from a group of serum proteins like biotinylated albumin, biotinylated transferrin and biotinylated IgG.
10. 3. A product according to Claim 1 or Claim 2, in the form of a kit of reagents including the measured quantities of the components defined in Claim 1 or Claim 2 in a form suitable for intravenous administration.
15. 4. A product according to Claim 3, in which the measured quantity of the component a) is between 0.5 and 10 mg.
20. 5. A product according to Claim 3, in which the measured quantity of the component b) constitutes a molar excess of from 10 to 100 times the measured quantity of the component a).
25. 6. A product according to Claim 3, in which the measured quantity of the component b) is from 5 to 100 mg.
30. 7. A product according to Claim 3, in which the component c) is radiolabelled or ready to be radiolabelled with a radioisotope selected from one of the following groups: a) Fe-52, Mn-52m, Co-55, Cu-64, Ga-67, Ga-68, Tc-99m, In-111, I-123, I-125, I-131; b) P-32, Sc-47, Cu-67, Y-90, Pd-109, Ag-111, I-131, Pm-149, Re-186, Re-188, At-211, Pb-212, Bi-212.
35. 8. A product according to Claim 3, in which the component c) is conjugated with a paramagnetic agent selected from gadolinium, iron and manganese.
40. 9. A product according to Claim 3, in which the component c) is conjugated with a cytotoxic agent selected from ricin, Adriamycin or cis-platinum.
45. 10. A product according to Claim 3, in which the component c) is constituted by biotinylated liposomes including a chemotherapeutic agent.
50. 11. A product according to Claim 3, in which the

measured quantity of the component b) is from 0.1 to 5 mg.

12. A method of fixing selectively an agent selected from the group consisting of radioactive isotopes, paramagnetic agents and cytotoxic agents to target tumour cells in a mammal suffering or suspected to be suffering from solid tumours, characterised in that it includes the sequential, intravenous administration of measured quantities of the components a), b) and c) as defined in Claim 1.

13. A method according to Claim 12, in which the measured quantity of the component a) is in a proportion of from 0.007 to 0.14 mg/kg of body weight and in which the quantity of the component b) administered constitutes a molar excess in comparison with the quantity of the component a) administered, the component b) being administered long enough after the administration of the component a) for the biotinylated antibodies which are not fixed to the tumour as a tumour-associated antigen/biotinylated antibody complex to be catabolised.

14. A method according to Claim 13, in which the measured quantity of the component b) is administered at least 24 hours after the administration of the component a) and constitutes an excess of from 10 to 100 times the quantity of the component a).

15. A method according to Claim 14, in which the component b) is administered in two successive steps:

- i) 1/5 of the dose in 3 ml of physiological solution injected rapidly in a bolus;
- ii) 4/5 of the dose in 100 ml of physiological solution injected slowly (15-30 minutes) 5-15 minutes after the injection of the first dose.

16. A method according to Claim 14, in which the component c) is administered at least 24 hours after the administration of the component b), and including, before the administration of the component c), the administration of a biotinylated agent d) capable of remaining in the plasma for a certain period of time and capable of binding and removing the circulating avidin not fixed to the tumour cells.

17. A method according to Claim 16, in which the component d) is selected from a group of serum proteins like biotinylated albumin, biotinylated IgG and biotinylated transferrin,

and is administered from 3 to 15 minutes before the administration of the component c).

18. A method according to any one of Claims 12 to 17, in which the component c) is conjugated with a paramagnetic agent and in which, after the component c) has been administered, the paramagnetic agent is located by NMR.

19. A method of reducing the level of the background during the in vivo detection by scintigraphy, intrasurgery location or NMR of complexes of tumour-associated antigen/biotinylated monoclonal antibody/avidin/biotin conjugated with a radioactive or paramagnetic agent, formed in vivo by the sequential administration of a biotinylated monoclonal antibody, avidin, and biotin conjugated with a radioactive or a paramagnetic agent, including the administration of a measured quantity of biotinylated albumin before the biotin is administered.



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PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention
shall be considered, for the purposes of subsequent
proceedings, as the European search report

EP 91 12 1419

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 5)
X, Y	EP-A-0 251 494 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) * Page 2, lines 31-39; page 3, lines 31-35; claim; abstract * ---	1-19	A 61 K 49/02 A 61 K 47/48 A 61 K 49/00
X	THE JOURNAL OF NUCLEAR MEDICINE, vol. 28, no. 8, August 1987, pages 1294-1302, New York, US; D.J. HNATOWICH et al.: "Investigations of avidin and biotin for imaging applications" * Whole document * ---	1-19	
X, Y	WO-A-8 702 893 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) * Page 23, lines 18-28; page 59, example 12; claims 31,118 * ---	1-19 -/-	
INCOMPLETE SEARCH			
<p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>Remark: Although claims 12-19 are directed to a method of treatment of (diagnostic method practised on) the human/animal body (Article 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition.</p>			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	31-03-1992	BERTE M.J.	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document	T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document		



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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	<p>CHEMICAL ABSTRACTS, vol. 110, no. 18, abstract no. 160325c, Columbus, Ohio, US; V.S. TRUBETSKOI et al.: "Immunoliposome complexes: preparation, characterization and directed delivery to the human endothelial cell culture", & BIOL. MEMBR., 6(2), 143-8 * Whole abstract *</p> <p>-----</p>	1,10	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)